

# Topologically Restricted Appearance in the Developing Chick Retinotectal System of Bravo, a Neural Surface Protein: Experimental Modulation by Environmental Cues

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**Abstract.** A novel neural surface protein, Bravo, shows a pattern of topological restriction in the embryonic chick retinotectal system. Bravo is present on the developing optic fibers in the retina; however, retinal axons in the tectum do not display Bravo. The appearance of Bravo in vitro is modulated by environmental cues. Axons growing out from retinal explants on retinal basal lamina, their natural substrate, express Bravo, whereas such axons growing on collagen do not. Retinal explants provide a valuable system to characterize the mechanism of Bravo restriction, as

well as the cellular signals controlling it. Bravo was identified with monoclonal antibodies from a collection generated against exposed molecules isolated by using a selective cell surface biotinylation procedure. The NH<sub>2</sub>-terminal sequence of Bravo shows similarity with L1, a neural surface molecule which is a member of the immunoglobulin superfamily. This possible relationship to L1, together with its restricted appearance, suggests an involvement of Bravo in axonal growth and guidance.

WHEN neural networks are generated during embryogenesis, axons migrate along defined routes to their targets. The guidance of the axons depends on their interaction with the environment. Molecules exposed on the axonal surface and on the growth cone are assumed to participate in this interplay, a precondition of axonal outgrowth, fasciculation, and pathfinding (for reviews see Edelman, 1985; Jessell, 1988; Rathjen, 1988; Harris and Holt, 1990). An involvement of surface molecules in axonal outgrowth (Lagenaur and Lemmon, 1987; Bixby and Zhang, 1990; Chang et al., 1990; Furley et al., 1990), fasciculation (Rathjen et al., 1987a,b), and guidance (Matsunaga et al., 1988) has indeed been demonstrated. Many of these surface glycoproteins share structural features, such as the HNK-1 epitope (Pesheva et al., 1987; Gennarini et al., 1989) and immunoglobulin-like domains (Cunningham et al., 1987; Harrelson and Goodman, 1988; Moos et al., 1988; Ranscht, 1988; Seeger et al., 1988; Bieber et al., 1989; Brümmendorf et al., 1989; Gennarini et al., 1989; Furley et al., 1990). These domains supposedly are involved in cell adhesion and are indicators of the evolutionary development of cell interactions (Edelman, 1984, 1985, 1987; McClay and Ettensohn, 1987; Rutishauser et al., 1988; Schachner et al., 1988; Williams and Barclay, 1988).

Molecules involved in axonal guidance are expected to be expressed early in development, when neural connections are forming, under temporal or topological restriction (Raper et al., 1983a,b; Bastiani et al., 1984; Dodd and Jessell, 1988; Harrelson and Goodman, 1988). Indeed, fasciculins I and II in the grasshopper (Bastiani et al., 1987) and TAG-1 in the rat (Dodd et al., 1988) show a restricted pattern of expression. The mechanisms controlling such restricted expression are still not well understood.

The retinotectal system of the chick embryo is especially suited for studies of axonal outgrowth and navigation. Whole organ preparations, as well as retinal explant cultures, allow manipulation of the axonal environment under conditions that maintain the natural recognition properties of the optic fibers (Halfter et al., 1981; Bonhoeffer and Huf, 1982; Walter et al., 1987a,b).

This project was aimed at the identification and characterization of molecules involved in the interactions between axons and their environment, using monoclonal antibodies specifically made for this purpose. Optic tecta of chick embryos at day 8 of development (E8)<sup>1</sup> were chosen as a source of antigen. At this stage, the optic fibers massively invade the optic tectum but most have not reached their targets (Crossland et al., 1975; Rager, 1980). Thus, both inter-

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1. *Abbreviations used in this paper:* E8, embryonic day 8 of development; RT, room temperature.

active partners of the retinotectal system, the optic fibers and their tectal targets, are found together in a single structure. Assuming that molecules participating directly in cell interactions are located on the cell surface or in the extracellular matrix, exposed molecules in the optic tectum containing growing optic fibers were specifically biotinylated (Boxberg et al., 1990). The labeled molecules were isolated by avidin-affinity chromatography and further fractionated according to size by HPLC. mAbs were raised against each of the fractions, yielding a large collection of antibodies specific for surface molecules. A detailed description of the method will be presented (Kayyem, J. F., J. M. Roman, D. B. Teplow, E. J. de la Rosa, U. Schwarz, and W. J. Dreyer, manuscript in preparation). The molecules recognized by different mAbs were designated using the international phonetic alphabet (Alpha, Bravo, Charlie, etc.), since the Greek alphabet is already heavily used in naming other proteins.

Bravo is one of the neural surface compounds found with those mAbs. Some biochemical and biological characteristics of this molecule will be described in this article.

## Materials and Methods

### Chick Embryos

All the experiments were performed with White Leghorn embryos of the indicated ages, obtained by incubation of fertilized eggs at 38.7°C.

### Immunochemical Methods

**Purification of Antigen by Immunoaffinity Chromatography.** Before its immobilization on a column, the antibody was purified from mouse ascites, either by HPLC on a TSK DEAE-5PW column (Bio-Rad Laboratories, Munich, Germany), according to Deschamps et al., 1985, or by chromatography on a CM Affi-Gel Blue column (Bio-Rad Laboratories), according to the manufacturer's instructions.

The purified mAb (25 mg) was bound to 10 ml of Affi-Gel 10 (Bio-Rad Laboratories), as recommended by the manufacturer, packed in a column which, before use, was run once with the various buffers to be used for antigen purification (see below).

An extract from PI chick whole brain was used as an antigen source. Brains were washed three times with 3 ml per brain of labeling buffer (140 mM NaCl, 5 mM KCl, 5 mM glucose, 7 mM NaHCO<sub>3</sub>, 1.5 mM MgSO<sub>4</sub>, 1.5 mM CaCl<sub>2</sub>, and 0.5 µl/ml aprotinin, 2 mg/ml iodoacetamide, 0.2 mg/ml PMSF, and 50 µg/ml soybean trypsin inhibitor as protease inhibitors) and homogenized in 3 ml per brain of Nicholson lysis buffer (10 mM Hepes, pH 7.5, 140 mM NaCl, 4 mM EDTA, 2.5% (wt/vol) NP-40 (Calbiochem-Behring, Corp., Frankfurt am Main, Germany), 2.5% (wt/vol) Zwittergent 3-14 (Calbiochem-Behring Corp.) (Updyke and Nicholson, 1984) supplemented with azide and protease inhibitors (as above). After centrifugation (50,000 g for 30 min) the supernatant from 100 brains was pumped onto the column with immobilized antibody at a flow rate of 10 ml/h. The column was then washed at a flow rate of 50 ml/h with (a) 100 ml of 20 mM Tris-HCl, pH 8.0, containing 140 mM NaCl, 0.5% (wt/vol) NP-40, and 0.5% (wt/vol) Zwittergent 3-14; (b) 50 ml of 50 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl and 0.5% (wt/vol) NP-40; (c) 50 ml of 50 mM Tris-HCl, pH 9.0, containing 0.5 M NaCl and 0.1% (wt/vol) NP-40. The antigen was finally eluted with 150 ml of 50 mM triethanolamine, pH 11.5, containing 150 mM NaCl and 0.1% (wt/vol) NP-40. 1.6-ml fractions were collected in tubes containing 0.4 ml of 1 M Tris-HCl, pH 6.7. The antigen in the fractions was identified by a dot blot assay (see below). The antigen peak was pooled and concentrated-dialyzed by six cycles of centrifugation (1,500 g for 30 min) in Centriprep 30 (Amicon Corp., Witten, Germany), with PBS containing 0.1% (wt/vol) NP-40. The antigen was concentrated by a factor of 15 and the elution buffer was diluted 10<sup>3</sup> times. In the case of Bravo, the final concentration of antigen was 30 µg/ml, as determined by silver staining of SDS gels in comparison with standards. The final yield was 250–300 µg of protein from 100 brains. The column was regenerated by washing with 200 ml of 20 mM Tris-HCl, pH 8.0, containing 140 mM NaCl, and 0.025% (wt/vol) azide.

**Protein Blots.** For dot blots (Hawkes et al., 1982), 1–10 µl of the sam-

ple to be analyzed was spotted onto nitrocellulose and dried. To reduce background staining, the nitrocellulose was kept for at least 2 h at RT with 5% (wt/vol) powdered skim milk and washed twice with PBS before immunostaining. Hybridoma culture supernatant (1:4 dilution), mouse ascites (1:2,000 dilution), or rabbit antiserum (1:25,000 dilution) were incubated with the blot for 1 h at RT. For all dilutions, and three washings between incubations, PBS containing 0.1% (vol/vol) Tween 20 was used. Finally, the blots were incubated for 1 h at RT with a 1:1,000 dilution of peroxidase-coupled second antibody and stained with 4-chloronaphthol (0.5 mg/ml) and 0.015% (vol/vol) of H<sub>2</sub>O<sub>2</sub> in PBS.

For Western blots, the samples were fractionated by electrophoresis (SDS-PAGE; 6% gel) and transferred to nitrocellulose in a semi-dry system as described by Kyhse-Andersen (1984). The Western-blots were treated as described above for the dot-blots.

### Protein Sequencing

NH<sub>2</sub>-terminal protein sequence information was gathered using a protein sequenator (model 477A; Applied Biosystems, Foster City, CA) with on-line PTH analysis (model 120A; Applied Biosystems). Protein antigens (30–200 pmol), purified by affinity chromatography as described above, were electroblotted onto polyvinylidene difluoride membrane after SDS-PAGE (Matsudaira, 1987).

### Immunohistochemical Methods

**Tissue Sections.** Chick embryos were sectioned with a cryomicrotome after fixation of the tissue. For small embryos, up to E8, the standard fixation was by overnight immersion in 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer, pH 7.0. Larger embryos were first perfused with fixative (as above); then tissue pieces were dissected and further fixed by overnight immersion in fixative. Alternatively, the tissues were fixed by overnight immersion in 2% (wt/vol) TCA. Routinely, the fixed tissue was infiltrated with 30% (wt/vol) sucrose in PBS before sectioning.

For staining with single antibodies, the sections were incubated sequentially with (a) 15% (vol/vol) normal goat serum in culture medium for 20 min at RT; (b) mAb, hybridoma culture supernatant (undiluted), or mouse ascites (1:1,000 dilution) or rabbit polyclonal antiserum (1:20,000) for 45 min at RT; (c) biotinylated second antibody (anti-mouse or anti-rabbit of 1:200 dilution) for 30 min at RT; and (d) fluorescein-Streptavidin (1/200 dilution) for 30 min at RT. The washing steps between incubations, as well as the dilutions were with PBS containing 0.2% (wt/vol) BSA. The sections were mounted for epifluorescence microscopy.

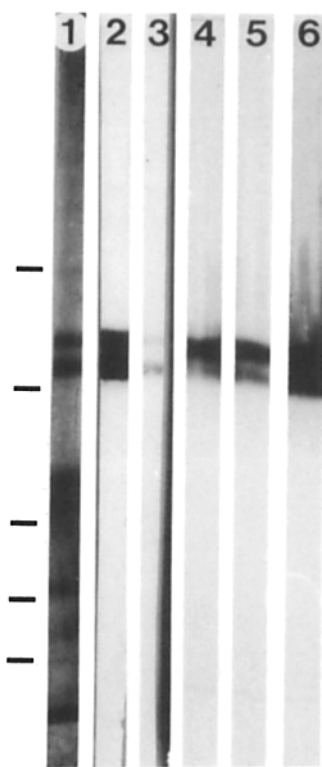
For double staining, mAb anti-G4/L1 purified by HPLC (Deschamps et al., 1985) was labeled with biotin, according to Clark and Todd (1982). Biotin X-NHS (Calbiochem-Behring Corp.) as a freshly prepared 20 mg/ml solution in DMSO was added to the antibody in PBS (biotin/mAb; 1:7; wt/wt) and incubated for 2 h at RT with vigorous shaking. Excess biotin was reacted with a 10-fold excess of glycine (30 min at RT). The mixture was directly used for staining. Sections were double stained as follows: (a) 15% (vol/vol) normal goat serum for 20 min at RT; (b) mAb anti-Bravo (culture supernatant or a 1:1,000 dilution of mouse ascites) for 45 min at RT; (c) fluorescein-labeled anti-mouse second antibody (1:80 dilution) for 30 min at RT; (d) normal mouse serum (1 mg/ml) for 30 min at RT; (e) biotinylated mAb anti-G4/L1 (5 µg/ml) for 45 min at RT; and (f) Texas red-Streptavidin (1/100 dilution) for 30 min at RT. The stained sections were screened in an epifluorescence microscope, with either fluorescein or rhodamine filter.

**Whole Mounts.** Retinas removed from chicken eyes were flattened out on a filter, with the basal lamina (inner part) up, as described by Halfter et al. (1983). Tectal whole mounts were prepared by dissecting the tecta out of the rest of the brain and spreading the isolated tecta flat on a filter, with the ventricular surface (inner part) down, similar to the retinal whole mounts (Kröger and Schwarz, 1990).

For EM, E6 retinal whole mounts were pre-fixed in 4% (wt/vol) paraformaldehyde for 4 h at RT and stained as described for tissue sections, except that incubation and washing times were doubled and that, in the last step, the whole mounts were incubated with peroxidase-Streptavidin (1:100 dilution) for 1 h at RT and reacted with diaminobenzidine (0.5 mg/ml) and H<sub>2</sub>O<sub>2</sub> (0.02% vol/vol) in PBS with Co<sup>2+</sup> and Ni<sup>2+</sup> enhancement. The tissue was postfixed with 2.5% (vol/vol) glutaraldehyde for 1 h at RT and processed for EM.

Tectal whole mounts were stained with mAbs as described for tissue sections, but with doubled incubation times. For staining native tissue, the whole mounts were first incubated with the antibody, then fixed with 4% (wt/vol) paraformaldehyde for 2 h at RT, and then stained.

**Explant Cultures.** Retinal explant cultures (see below) were stained as



**Figure 1.** Biochemical characterization of Bravo. Shown are Western blots of immunoaffinity-purified Bravo antigen stained with either gold (lane 1), mAb anti-Bravo (lane 2), or mAb L2 (lane 3). In extracts from E8 retina (lane 4), E8 whole brain (lane 5), and P1 whole brain (lane 6), Bravo was identified by Western blot and immunostaining with mAb anti-Bravo. The antigen-antibody complexes were revealed using the peroxidase procedure. The marks at the left margin indicate the position of molecular mass standards of 180, 116, 84, 58, and 48.5 kD.

described for tissue sections. The tissue was fixed (30 min at RT with 4% paraformaldehyde (wt/vol) either before the incubation with the mAb, or after, when living explants had been stained.

### Embryo Manipulations

**Tracing of the Optic Fibers.** The optic fibers were traced from the eye to the tectum by injection of rhodamine into the eye (Thanos and Bonhoeffer, 1983). A lateral window was opened in the shell of fertilized eggs after a 5-d incubation. The embryonic membranes were opened to allow access to the right eye, into which 1  $\mu$ l of a freshly prepared rhodamine solution was injected (1 mg of rhodamine dissolved in 2  $\mu$ l of DMSO, diluted with 100  $\mu$ l PBS and clarified by centrifugation) with a glass capillary. After the manipulation, the window was covered with a petri dish (35 mm), sealed with silicone and further incubated for a minimum of 30 h before whole mount tecta (tectum contralateral of the injected eye) or tissue sections were prepared.

**Eye Enucleation.** Both eyes were removed from E2 chick embryos (Thanos and Dütting, 1988). 2 ml of the egg white was extracted from the egg with a syringe and a lateral window was opened to give access to the embryo, which was contrasted by injecting India ink underneath it. The optic vesicles were removed with tungsten needles and the egg was sealed with a petri dish cover (35 mm) and silicone and allowed to further develop in the incubator. At the desired stage, embryos were prepared for tissue sections.

### Explant Cultures

Explant cultures from E6 retinæ were prepared as described by Halfter et al. (1983). The retina, mounted flat on a membrane filter, was cut in strips

with a tissue chopper set at 0.275 mm and cultured either on collagen gel or on retinal basal lamina, in DME/F12 medium (Gibco-BRL, Eggenstein, Germany) with 10% heat-inactivated FCS, 2% chick serum, and 50  $\mu$ g/ml gentamicin. After 2 d in culture, the explants were stained. The collagen gel substrate was prepared from rat tail collagen, as described (Halfter et al., 1983). The basal laminae used as growth substrate were prepared from E7 retinæ as described by Halfter et al. (1987). The basal lamina was fixed on petriperm plates (Heraeus-Amersil, Inc., Osterode, Germany), exposing the part which, in the retina, contacts the optic fibers. Such preparations, as shown by Halfter et al. (1987) consist of the basal lamina and the endfeet of ventricular cells.

## Results

### Biochemical Characterization of Bravo Antigen

Bravo antigen was purified by immunoaffinity chromatography. Two components of 130 and 140 kD on SDS-PAGE (Fig. 1) were isolated from a 1-d-old chick (P1) brain extract on a column with immobilized anti-Bravo antibody. On Western blots of retina and brain extracts, mAb anti-Bravo again stains two components of 130 and 140 kD. The amount of both components of the Bravo antigen (relative to wet weight of tissue) increases with the time of embryonic development (Fig. 1). Both the 130- and 140-kD components cross-react with the mAb L2, specific for the HNK-1 epitope found in other molecules involved in cell interactions (Schachner et al., 1988). The 130- and 140-kD molecules have the same NH<sub>2</sub>-terminal sequence (Fig. 2), showing remarkable similarity to the L1 molecule, a member of a group of neural surface proteins that belongs to the immunoglobulin superfamily and contains fibronectin type III domains (Moos et al., 1988).

Bravo does not show immune cross-reactivity with the molecules G4, F11, or neurofascin (Rathjen et al., 1987a,b; Brummendorf et al., 1989) (data not shown). This, together with the partial protein sequence data (Fig. 2), excludes identity between Bravo and other known molecules found in fiber-rich regions of the developing chick embryo.

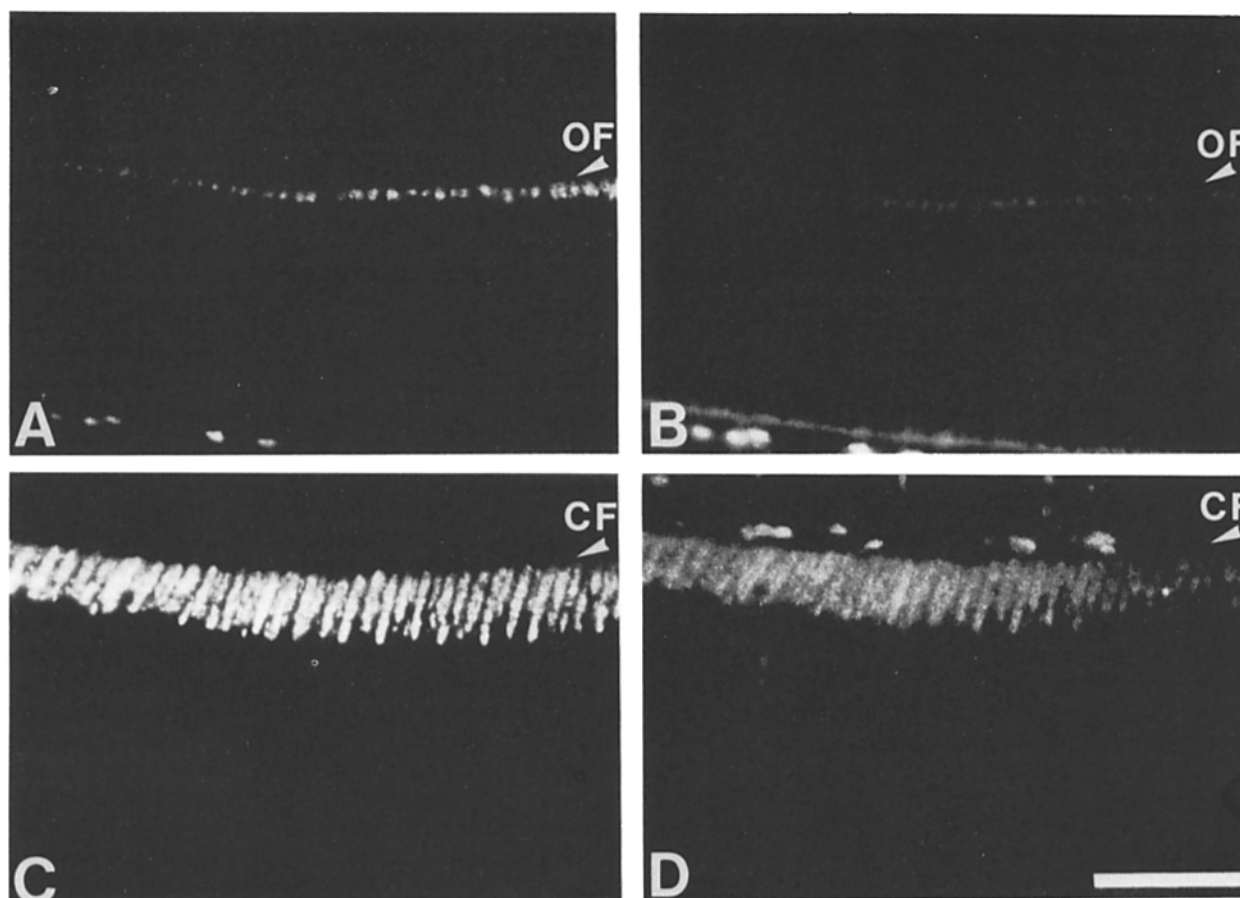
### Immunohistochemical Localization of Bravo in the Developing Chick Retinotectal System

mAb anti-Bravo stains all the fiber layers of the retina and the optic tectum as soon as they are morphologically identifiable. Bravo appears in parallel with G4, an early axonal marker which is considered to be the chick homologue of L1 (this molecule will be referred to here as G4/L1) (Rathjen et al., 1987b; Lauer et al., 1988). Fig. 3 illustrates the simultaneous presence of both antigens at embryonic day 6 (E6), when the optic fiber fascicles in the retina (Fig. 3, A and B) and the circumferential fiber fascicles in the tectum (Fig. 3, C and D) are already well formed (LaVail and Cowan, 1971; Crossland et al., 1975; Puelles and Bendala, 1978; Rager, 1980). A coincident appearance of G4/L1 and Bravo

L1: NH <sub>2</sub> -	I	I	Q	I	P	D	E	Y	G	H	H	V	L
G4: NH <sub>2</sub> -	I	I	T	I	P	P	E	Y	A	H	D	F	L
BRAGO: NH <sub>2</sub> -	L	T	V	P	L	D	S	L	?	E	?	L	S

**Figure 2.** Comparison of the NH<sub>2</sub>-terminal sequences of the axonal surface proteins L1, G4, and Bravo. The sequences of L1 and G4 have been taken from

Moos et al. (1988) and Rathjen et al. (1987b), respectively. The sequence of Bravo was determined as described in Materials and Methods. Identical residues are squared. Note also that several nonidentical residues are functionally similar and suggest relatedness (e.g., isoleucine/leucine, glutamate/aspartate).



**Figure 3.** Parallel appearance of Bravo and G4/L1 in the developing chick retinotectal system. Shown are double-stained tissue sections of E6 chick embryo retina (*A* and *B*, same section) and tectum (*C* and *D*, same section). The axons are labeled either with mAb anti-G4/L1 (*A* and *C*) or with mAb anti-Bravo (*B* and *D*). Both the Bravo and the G4/L1 molecule appear in the first fibers originating in the retina, the optic fibers (*OF*) in *A* and *B*, and also in the first fibers that develop in the tectum, the circumferential fibers (*CF*) in *C* and *D*. Bar, 100  $\mu$ m.

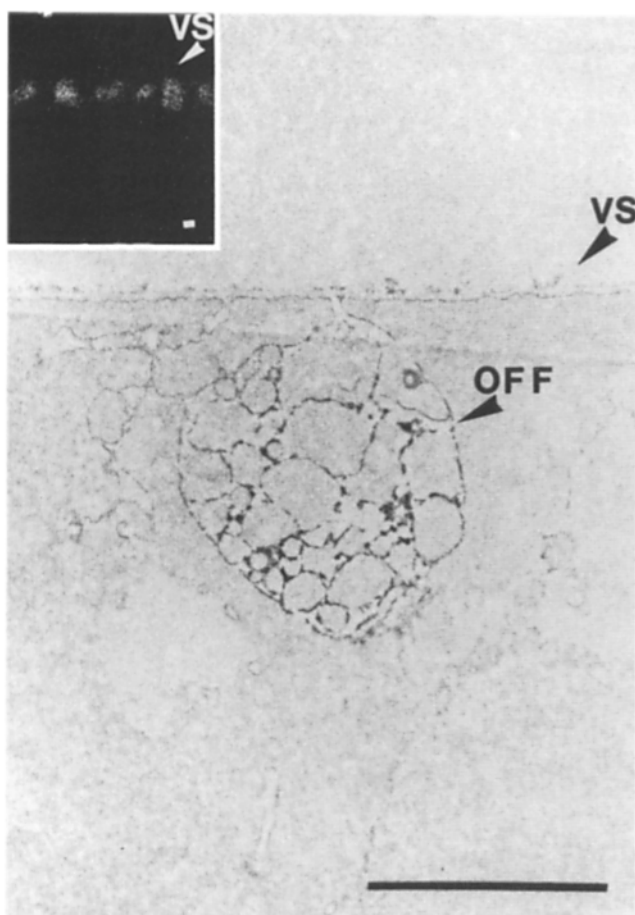
can be observed in the first emerging fibers along the whole neural tube and in cephalic vesicles at earlier stages of development (data not shown).

Since mAb anti-Bravo was raised against an antigen preparation enriched in surface molecules, we expected Bravo to be located on the cell surface or in the extracellular matrix. To investigate the precise location of this molecule, Bravo was also visualized in the retina by immunohistochemistry combined with EM. At E6, Bravo is clearly associated with the surface of the optic fibers in fascicles in the retina (Fig. 4). Furthermore, retinal explant cultures (see below) showed definitively that Bravo is exposed as an axonal surface molecule.

While the optic fibers in the retina carry Bravo, this molecule was not detected on retinal axons in the tectum. Optic fibers projecting to the tectum were traced by injection of rhodamine into the eye. The rhodamine-labeled fibers are visible when entering the tectum at the rostral pole of E6 whole mount tecta (Fig. 5, *A* and *C*). These fibers are not stained with mAb anti-Bravo (Fig. 5 *B*), but are stained with mAb anti-G4/L1 (Fig. 5 *D*). Circumferential fibers originating in the tectum, however, are stained by both mAbs (anti-G4/L1 and anti-Bravo), as shown by confocal microscopy of

E6.5 tectal whole mounts (Fig. 6). By optical sectioning, the staining by mAbs anti-G4/L1 and anti-Bravo was analyzed throughout the depth of the tissue. Circumferential fibers in deeper layers carry G4/L1 as well as Bravo, in marked contrast to the optic fibers present in upper levels, only stained by mAb anti-G4/L1. Computer image processing of the pictures did not reveal, at any amplification of the signal, fiber structures in the top levels of the tectum stained with mAb anti-Bravo. In contrast, E6.5 whole mount retinas show clear staining of the optic fascicles with mAbs anti-Bravo as well as anti-G4/L1 (data not shown). Neither the optic fibers in the whole mount retinas nor the circumferential fibers in the whole mount tecta show regional differences in the level of Bravo. When considered together, all observations indicate that, while G4/L1 is present uniformly along the optic fibers, Bravo is detected differently in the retina than in the optic tectum. A polyclonal antiserum generated against Bravo yielded identical results (data not shown).

At E8, most of the optic fibers have already reached the tectum and are forming the *Stratum opticum* (Crossland et al., 1975; Rager, 1980). Again, the optic fibers were not stained with mAb anti-Bravo, neither in E8 whole mount tecta nor in E8 tectal sections (data not shown), which sup-



**Figure 4.** Localization of the Bravo molecule in the optic fibers in the retina. The electron micrograph shows an optic fiber fascicle (OFF) transversally sectioned in the central region of an E6 retina. Bravo surrounds the axons as revealed by immunostaining. The retina was stained as a whole mount with mAb anti-Bravo (peroxidase procedure) before being processed for EM. The inset shows the position of axon fascicles in an equivalent section, fluorescence-labeled with mAb anti-Bravo, in the light microscope. VS indicates the vitreal (inner) surface of the retina. Bars, 2.5  $\mu$ m.

ports the observations made at earlier stages. On the contrary, the optic fibers were brightly stained by mAb anti-G4/L1 (data not shown). Consequently, the lack of staining by mAb anti-Bravo of the optic fibers in the tectum cannot result from a delayed expression of Bravo in comparison with G4/L1.

Bravo appears in the tectum in the same regions as the optic fibers, although these axons themselves are devoid of the Bravo molecule. To demonstrate that the appearance of Bravo in those optic regions is independent of the presence of optic fibers, tecta deprived of these fibers were studied. Fig. 7 shows sections of E8 tectum from embryos in which both eye vesicles had been removed at E2. These embryos lack the forming *Stratum opticum*. In contrast to the differences between normal and eyeless embryos stained for G4/L1 (Fig. 7, A and C), the general Bravo staining in the region of the forming *Stratum opticum* was not altered by the deprivation of fibers (Fig. 7, B and D). This suggests that tec-

tal structures, other than optic fibers, react with the mAb anti-Bravo. Ventricular cell processes and end-feet present in that region are possible candidates. In support of this, preparations of pial and tectal-limiting membrane (Kröger and Schwarz, 1990), including ventricular cell end-feet but free of fibers, show a strong Bravo reactivity (data not shown).

### Expression of Bravo on Axons of Retinal Explants

A topologically restricted appearance of Bravo in the optic fibers has been clearly shown. However, the morphological data do not give precise information about the molecular mechanisms underlying this phenomenon or the signals controlling it. A simple experimentally modifiable system was chosen for experiments to prove or disprove possible mechanisms and signals. Some of the early steps of neural development of the chick retinotectal system can be reproduced in explant cultures of embryonic retina (Halfter et al., 1981; Bonhoeffer and Huf, 1982; Halfter et al., 1983; Walter et al., 1987a,b). Therefore, this system was used to study the appearance of Bravo on axons growing on different substrates.

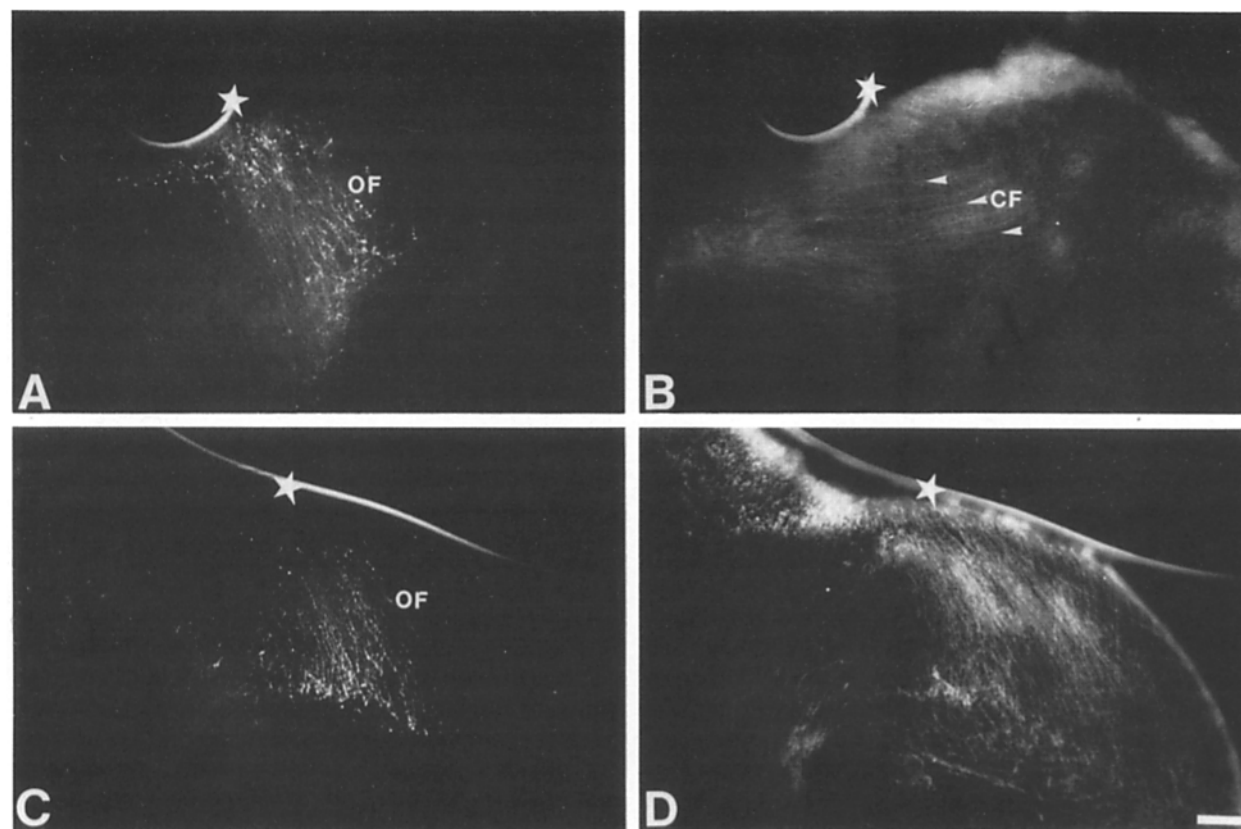
The basal lamina from the embryonic retina can be isolated, flattened out, and used as a substrate for outgrowing axons. This preparation consists of the inner basement membrane carrying the end feet of the ventricular cells (Halfter et al., 1987) and is very close to the natural substrate of optic fibers. Fibers grown on basal lamina indeed carry the Bravo molecule, as is the case in the embryonic retina (Fig. 8 B). The basal lamina preparation by itself does not show Bravo immunostaining. Intact living fibers are stained as well as fixed ones (data not shown), demonstrating the expected surface location of the Bravo molecule. This result was also reproduced with a polyclonal antiserum anti-Bravo (data not shown).

Most interestingly, however, the presence of Bravo on outgrowing retinal fibers was found to depend upon their environment. Very clearly, the fibers grown on collagen gel, which is a major structural component of the extracellular matrix but, in this case, not a natural substrate, carry very little, if any, Bravo (Fig. 8 D). Only close to the retina stripe, where some fibers fasciculate, a weak positive staining was detectable (data not shown). The lack of Bravo staining on fibers grown on collagen cannot result from an inaccessibility of the antibody to the fibers in the culture dish, since the mAb anti-G4/L1 stains them brightly (Fig. 8, A and C).

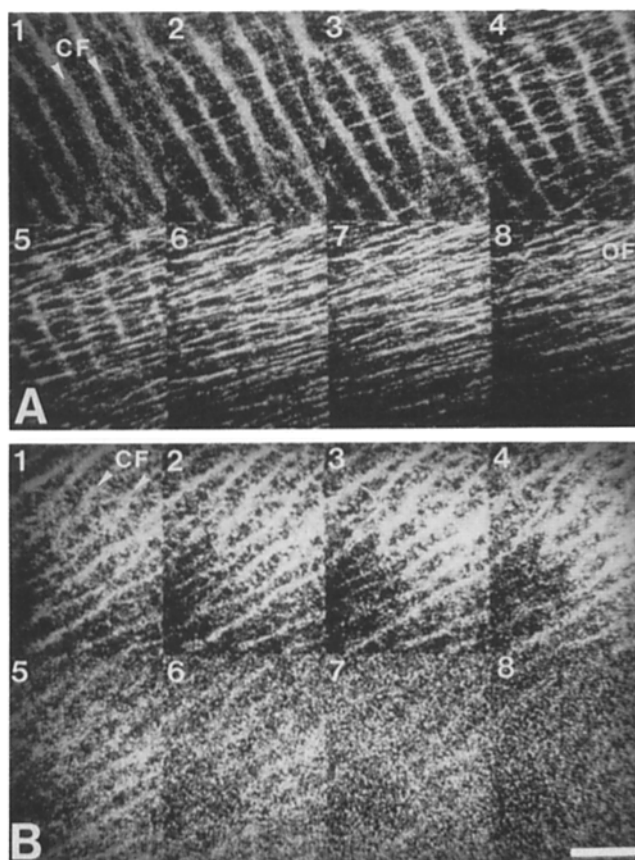
This result suggests that the appearance of Bravo on the surface of the optic fibers can be controlled by a signal associated with the retinal basal lamina, the natural substrate of these axons. The *in vitro* results are consistent with the *in vivo* situation, in which the optic fibers grow close to the basal lamina and display the Bravo molecule.

### Discussion

Increasing evidence is emerging that indicates that some of the molecules involved in the interaction of the axon with its environment during axonal navigation are subject to change and modulation during embryogenesis. Patterns of expression of such molecules define particular regions of an axonal pathway. In the grasshopper, for example, fasciclin is differentially expressed on subsets of axon fascicles (Bastiani et al., 1987). Fasciclin I is exposed in particular commis-



**Figure 5.** Absence of Bravo in the optic fibers when entering the tectum. Whole mount tecta showing rhodamine-labeled optic fibers (OF) in *A* and *C*, which are counterstained with mAb anti-Bravo (*B*), or with mAb anti-G4/L1 (*D*). Rhodamine was injected into the eye of E5 embryos. The subsequent staining with mAb anti-G4/L1 reveals a carpet of axons entering the tectum (*D*, same preparation as in *C*), which however is not detected with mAb anti-Bravo (*B*, same preparation as in *A*). Some tectal circumferential fibers in a deeper focal plane (CF) in *B* are stained with mAb anti-Bravo. The rostral poles of the tecta are indicated by stars. Bar, 100  $\mu$ m.

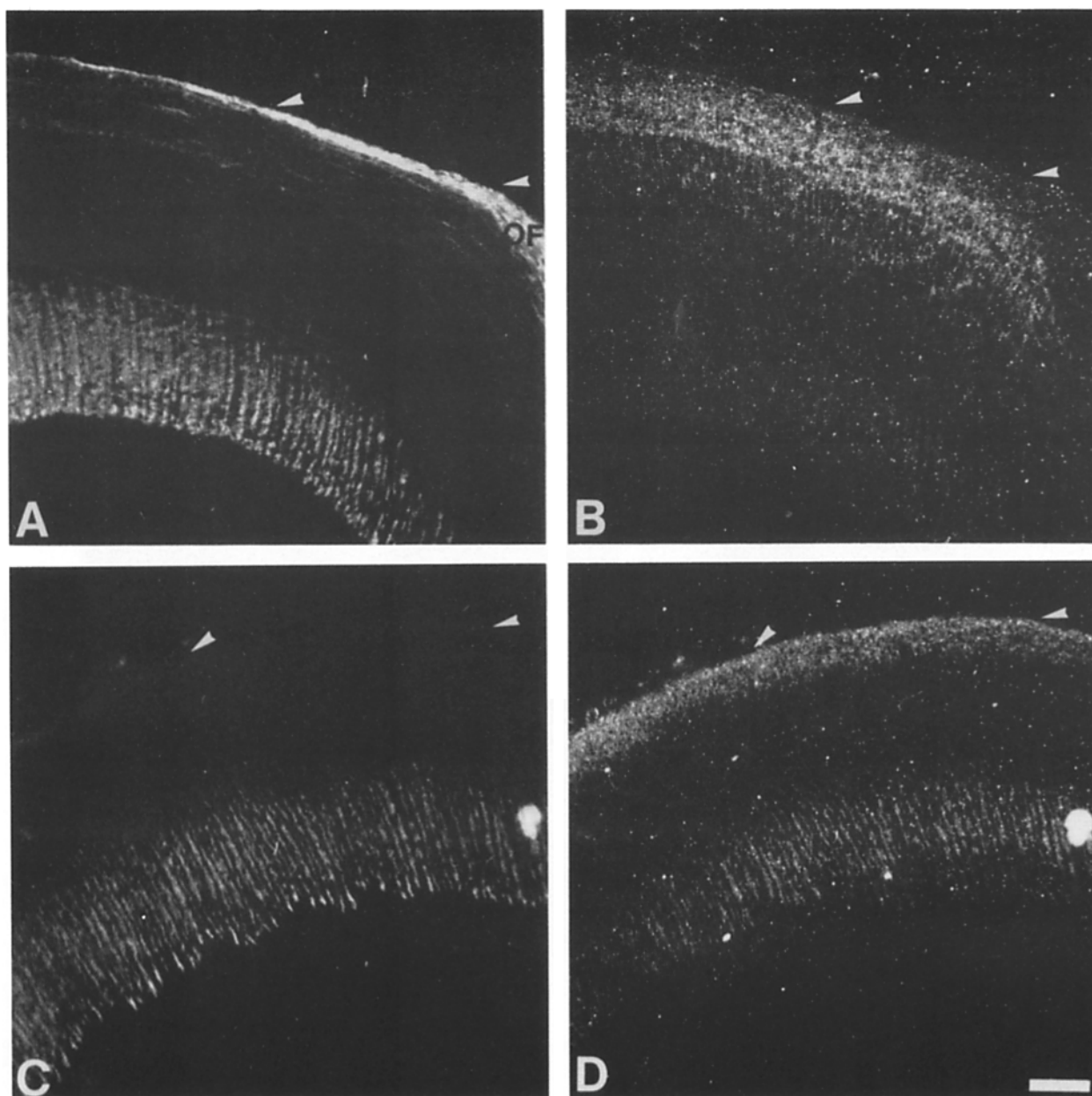


sural pathways, whereas fasciclin I is replaced by fasciclin II in longitudinal axon bundles. Quite similarly, in the rat embryo the glycoprotein TAG-1 appears transiently on subsets of spinal cord axons in commissural processes, whereas longitudinal fascicles express the molecule L1 (identical or closely related to Ng-CAM, NILE, G4/L1, and 8D9) (Dodd et al., 1988; Rathjen, 1988). The replacement of fasciclin I by fasciclin II, and of TAG-1 by L1, occurs in a single axon that expresses different proteins in defined regions of its pathway.

In addition to axonal surface protein shifts, the posttranslational modification of a single protein along axonal pathways has been observed. In the chick embryo, between days E5 and E10, the embryonic form of N-CAM, which is highly sialylated and weakly adhesive, as well as its adult form, less sialylated and highly adhesive, appear in parallel on different stretches along the trajectory of optic fibers (Schlosshauer et al., 1984). Whereas the perikaryon and intraretinal axons

**Figure 6.** Presence of Bravo and G4/L1 in the developing optic tectum. Whole mount tecta are shown, "optically sectioned" with a confocal microscope (system of Bio-Rad Laboratories, coupled with a Zeiss inverted microscope). The tecta (E6.5), labeled with mAb anti-G4/L1 (*A*) or with mAb anti-Bravo (*B*), were scanned (every 3  $\mu$ m) from the bottom (section 1) to the top (section 8). The circumferential fascicles (CF) in deeper layers (1–4) are stained with both antibodies whereas the optic fibers (OF) in the upper layers (5–8), readily stained with mAb anti-G4/L1, are not detected by mAb anti-Bravo staining. Bar, 100  $\mu$ m.





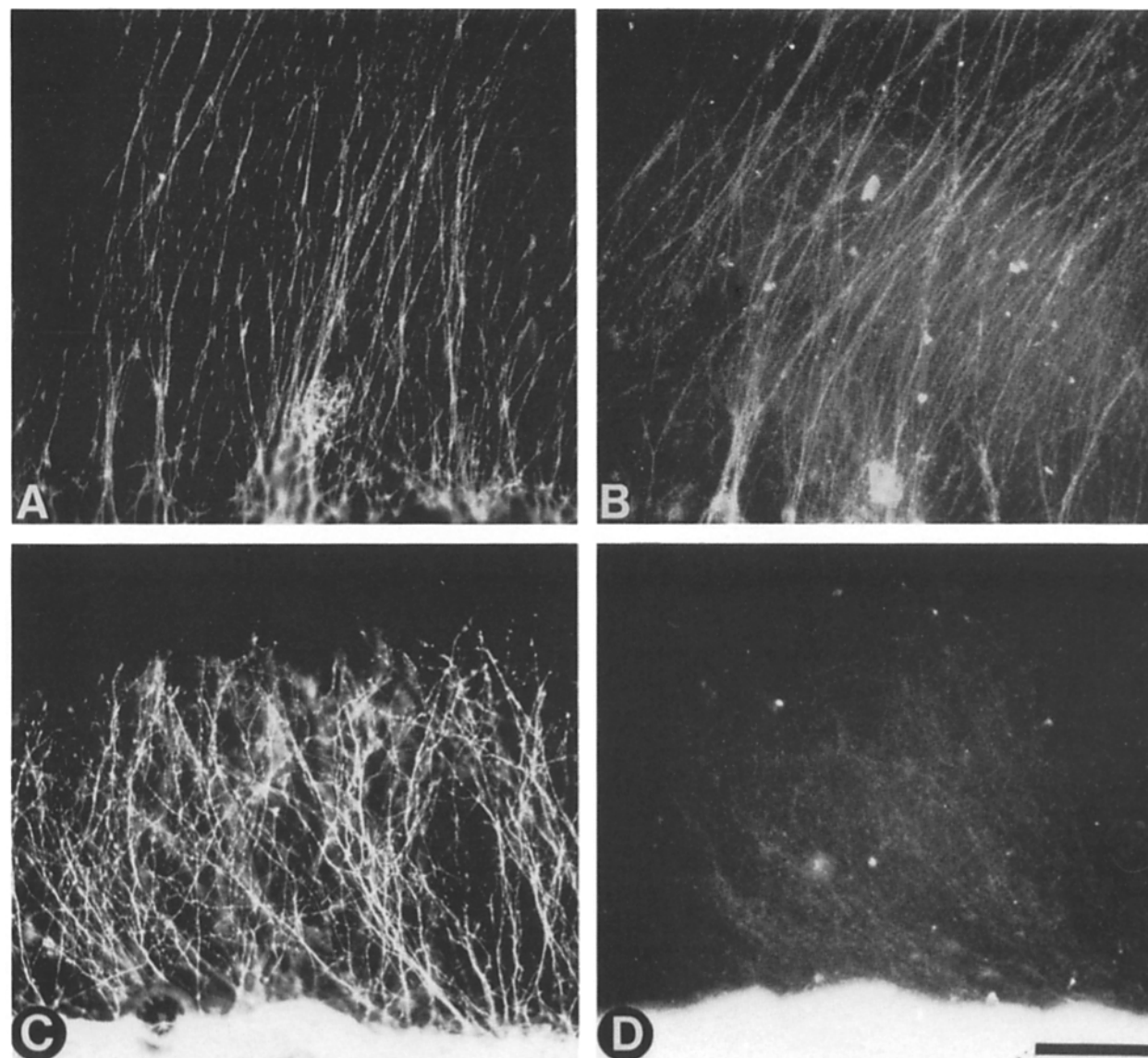
**Figure 7.** Presence of the Bravo molecule in the tectum deprived of optic fibers. Tectal sections are shown of a normal E8 embryo (*A* and *B*, same section), compared with those of an eyeless E8 embryo (*C* and *D*, same section), from which both optic vesicles had been removed at E2. The sections were stained with mAb anti-G4/L1 (*A* and *C*), which demonstrates the complete absence of optic fibers (*OF* in *A*) in the eyeless embryo (*C*). mAb anti-Bravo (*B* and *D*) reveals the presence of the Bravo molecule also in tecta devoid of optic fibers (*D*), indicating that Bravo is present in structures other than the optic fibers. A slight difference observed between normal and optic fiber-deprived tecta is due to the lag in development as a consequence of the manipulation during enucleation. The arrows indicate the position of the forming *Stratum opticum* in *A* and *B* and the corresponding position in *C* and *D*. Bar, 100  $\mu$ m.

carry the low sialic acid derivative, extraretinal axons expose the embryonic form rich in sialic acid.

As described here, another case of spatial restriction of surface compounds along axonal pathways is reflected by the appearance of the Bravo molecule along the retinotectal projection. Bravo appears as a surface protein early in development, at about the time when axons start to emerge from neuronal cells. Optic fibers within the retina expose the Bravo molecule (Figs. 3 *B* and 4), whereas, at the same stage of development, when entering the tectum, these axons are

devoid of Bravo or carry it in an undetectable form (Figs. 5 and 6). As opposed to G4/L1, whose distribution is relatively uniform along the optic fibers, Bravo shows a topologically restricted location in the chick retinotectal projection.

The mechanisms underlying the topologically restricted expression of molecules are not yet well characterized. The expression of TAG-1/L1 and of fasciclin I and II may be controlled either by an autonomous program of the neuron or by signals from the axonal environment. In the case of Bravo, the appearance of the molecule on the axonal surface de-



**Figure 8.** Substrate-dependent appearance of Bravo on fibers in retinal explant cultures. Shown are retinal explants grown for 2 d on retinal basal lamina (*A* and *B*) or on collagen gel (*C* and *D*). The explants were stained with mAb anti-G4/L1 (*A* and *C*) or with mAb anti-Bravo (*B* and *D*). The staining with mAb anti-G4/L1 is intense on fibers grown on either substrates (*A* and *C*). On the contrary, fibers grown only on retinal basal lamina are intensively stained with mAb anti-Bravo (*B*), in contrast to fibers grown on collagen (*D*). The explanted retina stripes are at the bottom of the pictures. The preparations of each mAb were photographed and printed under identical exposure conditions. Bar, 100  $\mu$ m.

pend, at least in vitro, on environmental cues. Axons grown on retinal basal lamina carry Bravo, in contrast to axons grown on collagen gel (Fig. 8). The precise nature of the environmental signals, of their topological control, and of the cellular response remains to be elucidated.

Many mechanisms could account for such a pattern of topological restriction. Differential synthesis and turnover, selective membrane insertion and stabilization by interaction with cytoskeletal or extracellular components, and differential release from the membrane are, in principle, equally plausible. The simplicity of the retinal explant culture system, as described in this paper, allows manipulations of the fiber environment and observation of its consequences on the expression of the Bravo molecule. The availability of such a

system is a prerequisite to analyze the cellular and molecular mechanisms involved in topological restriction. In addition, confocal microscopy together with image processing and analysis should allow a precise quantification of the amounts of Bravo.

The Bravo molecule is also found in neural tracts other than the optic fibers. This implies a role of the molecule beyond the retinotectal system. At present, a discussion of the function of Bravo has to remain speculative. The restricted pattern of expression during the formation of the retinotectal projection suggests a role in axonal navigation. Also the possible relationship of Bravo with L1 and with other members of the immunoglobulin superfamily, signals its involvement in cell-cell and/or fiber-fiber contacts. The possibility that



Bravo functions through a specific binding interaction with itself, with G4/L1, or with other molecules needs to be investigated.

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